The Disulphide Bonds of Insulin

BY A. P. RYLE, F. SANGER,* L. F. SMITH* AND RUTH KITAI
Department of Biochemistry, University of Cambridge

(*Received* 26 *November* 1954)

In order to deduce the unique structure of ox insulin it is necessary to know its molecular weight. In previous papers a value of 12 000 was assumed, since physical measurements suggested that this was the weight of the smallest unit that existed in solution. Recently, however, Harfenist & Craig (1952) have used a new chemical method and have found a value of approximately 6000. It is difficult to see how this result could be wrong and some recent physical measurements have shown that dissociation into units of molecular weight lower than 12 000 definitely occurs (Fredericq, 1953; Kupke & Linderstrøm-Lang, 1954). It may thus safely be concluded that the molecular weight of insulin is 5734, on the basis of condensation of the amino acids present with normal elimination of water. This molecule is composed of two polypeptide chains joined together by the disulphide bridges of three cystine residues. Treatment with performic acid splits the insulin to two fractions *A* and *B*, which are the oxidized forms of the glycyl and the phenylalanyl chain respectively (Sanger, 1949*a*). The sequence of amino acids in these two polypeptide chains has been determined by partial hydrolysis methods (Sanger, 1949*b*; Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*; Sanger, Thompson & Kitai, 1955) and is shown in Table 1. Fraction *A* contains four and fraction *B* two cysteic acid residues, originating from the three cystine residues of insulin. The purpose of the present study was to find which half-cystine residues are joined together in intact insulin.

For this it was necessary to identify peptides containing cystine residues and to determine their structure. The procedure used may be summarized as follows:

(1) Partial hydrolysis of insulin under conditions where the disulphide bonds remained intact.

(2) Fractionation of cystine peptides from one another. It was not, however, necessary at this stage to separate them from other peptides not containing cystine, since these were separated from the cysteic acid peptides during stage 4.

(3) Oxidation of cystine peptides to cysteic acid peptides.

(4) Fractionation of cysteic acid peptides.

(5) Identification of the cysteic acid peptides from the amino acids produced on hydrolysis. These peptides had already been obtained from partial hydrolysates of the fractions *A* and *B*, so that their structures were known and they could be completely identified from their hydrolysis products.

From the cysteic acid peptides produced the structure of the original cystine peptides could be deduced and hence the distribution of the disulphide bonds in insulin.

During stages (1) and (2) it was essential to avoid any re-arrangement of the disulphide bonds, and this was the chief difficulty in the work. Initially, hydrolysis was carried out in concentrated hydrochloric acid and the fact that it was impossible to interpret the results in terms of a unique structure for insulin suggested that a random re-arrangement of the disulphide bonds had taken place. This was confirmed by studies in model systems and the reaction has been further studied (Ryle & Sanger, 1955) and shown to occur in acid and neutral solution by different mechanisms. The reaction in neutral solution was catalysed by thiol compounds and could be prevented to some extent by thiol inhibitors such as *N*-ethylmaleimide (NEMI). It was thus possible to use proteolytic enzymes for partial hydrolysis and the position of one disulphide bridge was found in this way. Experiments using chymotrypsin and a crude pancreatic extract are described. However, no enzyme could be found that would break between the two half-cystine residues in positions *A* 6 and *A* 7 (Table 1) and acid hydrolysis had to be used to locate the remaining two disulphide bridges.

Various conditions of acid hydrolysis were tried but invariably led to re-arrangement until it was found that, unlike the neutral reaction, the acid one was inhibited by thiol compounds. It also appeared to be slower in H_2SO_4 than in HCl of the same concentration (Ryle & Sanger, 1955). A rather long time of hydrolysis was necessary to avoid the production of large amounts of peptides containing the *A* 6 . *A* 7 sequence (Table 1), as they do not provide the information required to establish the position of the bridges.

It was also necessary to avoid re-arrangement during the fractionation of the cystine peptides. Initially paper chromatography was used, but it was

* Members of the Scientific Staff of the Medical Research Council.

Table 1. *The structure of fractions A and B of oxidized insulin*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
					NH_2										NH_2			NH_2			NH_2									
Fraction *A* (Sanger & Thompson, 1953*a*, *b*; Sanger *et al.* 1955)	Gly	Ileu	Val	Glu	Glu	$CySO_3H$	$CySO_3H$	Ala	Ser	Val	$CySO_3H$	Ser	Leu	Tyr	Glu	Leu	Glu	Asp	Tyr	$CySO_3H$	Asp									
			NH_2	NH_2																										
Fraction *B* (Sanger & Tuppy, 1951 *a*, *b*)	Phe	Val	Asp	Glu	His	Leu	$CySO_3H$	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	$CySO_3H$	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala

difficult to obtain sufficient material, and frequently re-arrangement appeared to have occurred. The best results were obtained with paper ionophoresis and in the most recent experiments this method was used almost entirely.

The peptides with reference numbers starting *A* or *B* are those obtained in previous papers as follows: *A* 1, *A* 2, Sanger & Thompson (1953*a*); *A*p, *A*c, Sanger & Thompson (1953*b*); *B* 1, *B* 2, *B* 3, *B* 4, *B* 5, Sanger & Tuppy (1951*a*); *B*p, *B*c, *B*t, Sanger & Tuppy (1951*b*).

A preliminary communication of the present work was made by Sanger, Smith & Kitai (1954).

MATERIALS

The insulin used throughout this work was 6-times recrystallized cattle insulin (batch 9011 G), obtained from Boots Pure Drug Co., Nottingham. Crystalline chymotrypsin was obtained from Worthington Biochemical Sales Co., Freehold, New Jersey, U.S.A.

METHODS

Ionophoresis

The method used was essentially that of Michl (1951) in which the ionophoresis is carried out at high potential gradients in pyridine–acetic acid buffers on filter papers

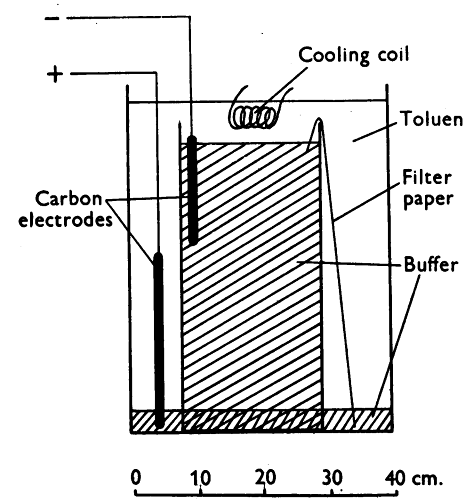


Fig. 1. High-voltage ionophoresis apparatus (Michl,1951).

completely immersed in toluene. This last prevents evaporation and cools the paper. It was found that sharper bands were obtained by this method than by other methods studied and the short times required for separations minimized the danger of breakdown or re-arrangement of cystine peptides during ionophoresis.

The apparatus, which is shown in Fig. 1, was made from two battery jars, one fitting inside the other. By having the level of the buffer solution higher in the cathode than in the anode vessel, electroendosmotic flow was to some extent counterbalanced by hydrostatic flow. Carbon rods were used for the electrodes and were connected to platinum wires underneath the toluene. Suitably shaped glass rods were used to prevent the paper from touching the sides of the vessels, and the whole apparatus was covered with a glass lid.

Since the buffer (pyridine–acetic acid) is somewhat soluble in the toluene and large volumes were involved, it was not convenient to keep changing the buffer for different experiments. Two units were therefore employed; the one contained buffer at pH 6·5 (10 vol. pyridine: 0·4 vol. acetic acid: 90 vol. water), the other at pH 3·6 (1 vol. pyridine: 10 vol. acetic acid: 89 vol. water). In some of the earlier experiments 0·2M acetic acid (pH 2·75) was used as the electrolyte. A paper up to 20 cm. wide could be used in the apparatus. The material to be fractionated was applied as a thin line across the dry paper to within about 1 cm. of each edge. It was allowed to dry and then laid on a glass plate with a glass rod underneath the line of application. The paper was wetted with the buffer to within about 1 cm. of the applied material and the buffer allowed to flow in towards the line of application from both sides, thus concentrating the material in a thinner line. It was essential to ensure that the buffer flowed in evenly and at the same rate from both sides and to be certain that the paper was completely wetted before putting in the apparatus. Where rather large concentrations of peptides are applied they sometimes wet very slowly. If a spot is not wetted by the buffer it is wetted by the toluene and causes an unevenness of flow. After blotting the paper to remove excess buffer it was put in the apparatus.

For most purposes a potential of 1500 v was applied. Using a 20 cm. wide Whatman no. 3 filter paper, a current of 60–70 mA was produced with the buffer at pH 3·6 and of 40–45 mA at pH 6·5. Owing to differences in design of the apparatuses the potential gradients produced were 29 and 33 v/cm. respectively. With no. 4 papers about half these currents are obtained. At the higher currents there is considerable heating, and it was necessary to have a cooling coil in the toluene. To find the position occupied by the peptides after ionophoresis, marker strips were cut from the paper and tested with a suitable dipping reagent, as below.

Ninhydrin. A 0·25% solution in acetone was used (Toennies & Kolb, 1951).

CN-*nitroprusside.* To test for the presence of cystine peptides the CN-nitroprusside test of Toennies & Kolb (1951) was used. This was found to be more sensitive than other tests investigated, though the colour was rather transitory, and it was necessary to record the position of the bands fairly rapidly.

Tyrosine test. To test for tyrosine peptides the method of Acher & Crocker (1952) was used.

Chymotryptic hydrolysis (*Expt. Ic*)

Insulin (100 mg.) was suspended in 10 ml. water and dissolved by the addition of NH_3 to give a final pH of 8. Chymotrypsin (4 mg.) and 1 mg. of NEMI were added and the mixture was incubated at 37° for 24 hr. The solution was then brought to pH 5·7 by the addition of dilute acetic acid. This produced a precipitate, which will be referred to as the 'chymotryptic core'. It is probably essentially the same as the 'core' studied by Butler, Dodds, Phillips & Stephen (1948) which was obtained from a chymotryptic hydrolysate by precipitation with trichloroacetic acid. The yield of 'core' was about 65 mg. It was further purified by dissolving in dilute NH_3 and precipitating at pH 5·7. In certain experiments the 'core' did not precipitate immediately on bringing the hydrolysate to pH 5·7 and it was necessary to concentrate to a smaller volume. However, once the precipitate had been obtained, it could not be redissolved near pH 5·7.

The soluble material from the hydrolysate was freeze-dried before being subjected to fractionation by ionophoresis.

Hydrolysis with a crude pancreatic extract (*Expt. Ix*)

The preparation used was a crude 'trypsin' (British Drug Houses, no. 557). It apparently contained several other enzymes, since several bonds which were outside the specificity range of trypsin or chymotrypsin were extensively hydrolysed.

Insulin (300 mg.) was dissolved in 18 ml. 0·01 N-HCl, 1 mg. of NEMI was added and the pH was adjusted to 7·8 by the addition of 0·1 N-NH_3. 1·2 mg. of the 'trypsin' preparation was then added in solution in a little water, and the pH was re-adjusted to 7·8. After 4 hr. incubation at 37° the pH had fallen considerably and was brought back to 7·8. After a further 17 hr. incubation the pH was 6·8 and the solution was very cloudy. The digest was then brought to pH 6 by the addition of 0·2 N acetic acid and centrifuged to remove the precipitated material. On concentrating the supernatant solution *in vacuo* a further small precipitate appeared, but this was ignored, and the whole of the concentrate was applied across a 25 cm. wide strip of Whatman no. 3 paper and was subjected to ionophoresis at pH 6·5 (in buffer containing approx. 10^{-3}M NEMI) at 1500 v for 12 hr.

Acid hydrolyses

A number of different experiments were carried out using acid with various conditions of hydrolysis and fractionation. Two such experiments will be described which incorporate the various techniques used and results obtained. In the first (*I*1) 10N-H_2SO_4 at 100° was used and the peptides were separated into two groups (*I*1α and *I*1β) by ion-exchange chromatography and were then fractionated by a number of different ionophoreses. In the second (*I*2) the hydrolysis was carried out at 37° and the neutral cystine peptides were separated by two-dimensional ionophoresis.

*Experiment I*1. The 'chymotryptic core' (100 mg.) was dissolved in a mixture of 5 ml. 20N-H_2SO_4, 3 ml. glacial acetic acid and 2 ml. water. Thioglycollic acid (3 μl.) was added and the mixture heated on a boiling-water bath for 45 min.

Removal of the H_2SO_4 with Ba caused extensive losses of the cystine peptides and was much more conveniently carried out using the basic ion-exchange resin Amberlite IR-4B, 44–100 mesh/in. (manufactured by The Rohm and Haas Co., Philadelphia, U.S.A.) in the acetate form. A column was prepared 8 cm. high in a tube of radius 2 cm. having a tap at the bottom. The resin was equilibrated against 20% (v/v) aqueous acetic acid and the hydrolysate poured on the column. To prevent uneven flow the top half of the column was stirred with a long glass rod so that most of the H_2SO_4 was initially adsorbed at the top of the column. The column was then allowed to flow and was developed with 20% acetic acid. The effluent was collected till a drop on a filter paper no longer gave the ninhydrin reaction.

75–100 ml. was usually sufficient. In some earlier experiments 5% acetic acid was used for elution, but the cystine peptides were considerably retarded and a greater volume of effluent was required. The solution was concentrated to 10–20 ml. *in vacuo* in the rotary evaporator of Craig, Gregory & Hausmann (1950), using a solid CO_2–methyl cellosolve mixture to cool the condensing bulb. The thioglycollic acid and remaining acetic acid were extracted with ether and a small amount of NEMI (2–3 mg.) was added.

The solution was brought to pH 5 with aqueous NH_3 and put on a column of Amberlite IR-4B in the acetate form, which had been washed with distilled water until the effluent attained pH 3–4. The column was developed with water. Part of the hydrolysate was not retained by the column and was collected and freeze-dried ($I\,1\beta$). The peptides retained on the column were removed by eluting with 20% (v/v) aqueous acetic acid, the solution concentrated, extracted with ether and freeze-dried ($I\,1\alpha$).

Experiment I 2. Insulin (100 mg.) was dissolved in a mixture of 7 ml. 20 N-H_2SO_4 and 7 ml. acetic acid containing 6 μl. thioglycollic acid and kept at 37° for 17 days. H_2SO_4 was removed on Amberlite IR-4B as described in Expt. *I* 1 and the material freeze-dried in the presence of NEMI.

Two-dimensional ionophoresis. Hydrolysate from Expt. *I* 2 (50 mg.) was dissolved in 0·15 ml. water and applied on a line near to the anode end of a 18 cm. wide strip of no. 3 filter paper for ionophoresis at pH 3·6. A potential of 1500 v was applied for 5·5 hr. After drying the paper, a strip was cut from one edge and the position of the cystine peptides located by the CN–nitroprusside test. The appearance of this test strip is shown in Fig. 2. The most slowly

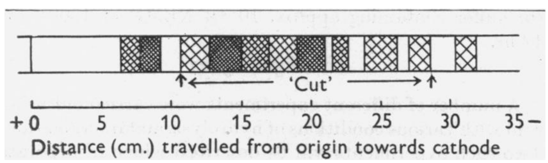


Fig. 2. Ionophoresis of partial hydrolysate of insulin (Expt. *I* 2): 50 mg./18 cm. wide no. 3 paper; pH 3·6; 29 v/cm.; 5·5 hr. In this and subsequent figures representing ionophoretic separations the different types of shading indicate the colour reaction used to locate the bands as follows: , ninhydrin reaction; , CN–nitroprusside reaction; , tyrosine reaction. The depth of shading represents the approximate strength of the colour reaction.

migrating material was largely free cystine, so was not fractionated further. The part of the paper between 10·6 and 28·6 cm. from the origin was then cut out and material eluted from it on to a second no. 3 filter paper as shown in Fig. 3. In this method one edge of the 'cut' was put in the trough of 10% acetic acid, which washed the peptide bands down near the 'front'. In order to keep this front as even as possible it was sometimes necessary to pipette drops of acetic acid on to the paper behind it. The other edge of the 'cut' was clamped down on the second paper with a glass plate so that only the extreme edge was in contact. When the acetic acid front had flowed into the second paper to form a band 1–2 cm. wide, the elution was stopped, the band dried off and then subjected to ionophoresis at pH 6·5 for 10 hr. using 1500 v. A small amount of NEMI was added to the buffer just before wetting the paper, to inhibit interchange reactions.

At the end of the ionophoresis the paper was hung up in air for about 10 min. to allow the surface toluene to drop off and evaporate. It was then laid on a flat sheet of glass. A dry sheet of no. 1 paper was laid on top of it and rapidly pressed down with another glass plate to make even contact and thus take a 'print'. Both papers were then dried. The no. 1 paper was treated with the CN–nitroprusside reagent, and from the positions of the spots, the cystine peptides could be located on the no. 3 paper, cut out and eluted.

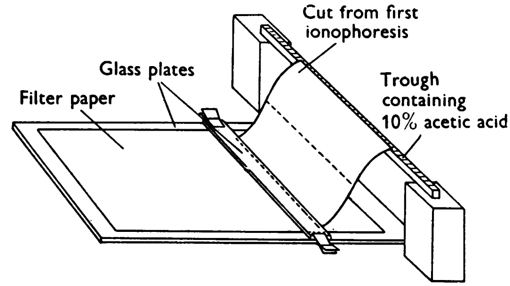


Fig. 3. Elution of peptide material during two-dimensional ionophoresis.

Structure of cystine peptides

To determine the structure of the cystine peptides they were oxidized with performic acid to the corresponding peptides of cysteic acid, which were then fractionated and subjected to complete hydrolysis. Previously the fractionation had been carried out by paper ionophoresis in acetic acid by which means all the cysteic acid peptides, except those containing a basic amino acid, could be completely separated from other peptides (Sanger & Thompson, 1953 *a*). More satisfactory results have now been obtained by carrying out the ionophoresis in pyridine–acetate buffers at pH 3·6. Here some fractionation of the individual cysteic acid peptides is obtained, which simplifies their identification.

The areas occupied by cystine peptides were cut out of the papers and eluted with 10% acetic acid. The eluates were put in small test tubes and taken to dryness in a desiccator. A few drops of a solution of performic acid prepared by mixing 1 vol. 33% (w/w) H_2O_2 with 9 vol. formic acid, were added. Oxidation was allowed to proceed for 15–30 min., a few drops of water were added and the solutions taken to dryness in a desiccator. Each fraction was then transferred to a polythene strip with a small volume of water and taken to dryness again to ensure removal of formic acid.

The residues were dissolved in about 10 μl. water and small portions (each about 2 μl.) were transferred with a capillary tube to a sheet of no. 4 paper near to the cathode end and subjected to ionophoresis at pH 3·6 for 2 hr. at 1500 v. The papers were dried and developed with ninhydrin.

From this preliminary ionophoresis some information could be obtained about the cysteic acid peptides present and it could be decided which fractions should be investigated further and at what concentration to carry out the ionophoresis. Thus, for instance, peptides containing two cysteic acid residues could readily be identified (see Figs. 14, 21) and since such peptides could not provide the required information, they were usually not investigated further. On the other hand, fractions giving two peptides each containing one residue of cysteic acid were of particular interest.

For further investigation of the cysteic acid peptides, the whole fractions were put on paper as a line and subjected to ionophoresis as above. A strip was cut from the edge and treated with ninhydrin to locate the position of the peptides, which were then cut out, eluted and hydrolysed. The resulting amino acids were identified by paper chromatography using phenol–0·3 % aqueous NH_3.

RESULTS

Chymotryptic hydrolysate (*Expt. Ic*)

Fig. 4 shows the distribution of peptide bands after ionophoresis of the soluble fraction (30 mg.) from the chymotryptic hydrolysate.

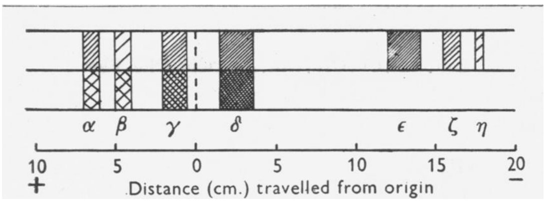


Fig. 4. Ionophoresis of peptides from soluble fraction of chymotryptic hydrolysate (Expt. *Ic*): 30 mg. on 18 cm. wide no. 3 paper; pH 6·5; 33 v/cm.; 2 hr.

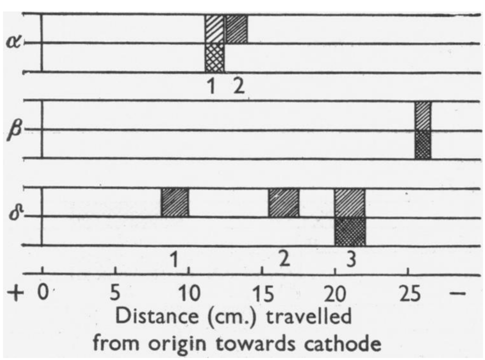


Fig. 5. Ionophoresis of fractions *Icα*, *Icβ* and *Icδ* (Fig. 4): no. 3 paper; pH 3·6; 29 v/cm.; 7 hr.

Band ε, which contained no cystine, was refractionated on a two-dimensional paper chromatogram using phenol–0·3 % NH_3/butanol–acetic acid. One main spot was present which proved to be Tyr.Thr.Pro.Lys.Ala (Table 2). Band ζ was similarly shown to be Thr.Pro.Lys.Ala and band η Pro.Lys.Ala. This last peptide had not been obtained from the chymotryptic treatment of fraction *B* (Sanger & Tuppy, 1951*b*). Its presence is surprising since it would not be expected from the known specificity of chymotrypsin.

Bands α, β and δ were further purified by ionophoresis at pH 3·6 (Fig. 5). Both δ1 and δ2 gave phenylalanine and tyrosine on hydrolysis. Band δ2 probably contains the free amino acids, since they move at the same rate on ionophoresis at pH 3·6. Band δ1 is probably a dipeptide Phe.Tyr, which would be expected to travel slower than the free amino acids. This dipeptide was not detected in the chymotryptic hydrolysate of fraction *B*, but might be expected to occur from the known specificity of chymotrypsin.

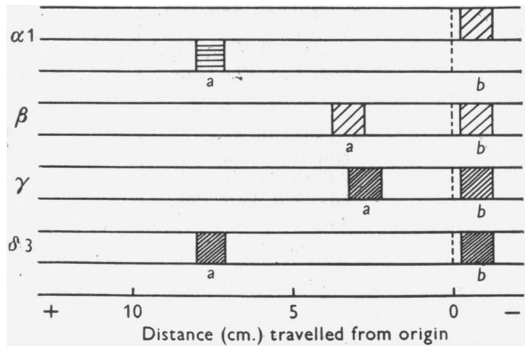


Fig. 6. Ionophoresis of oxidation products of bands α1, β, γ and δ3 (Figs. 4, 5): no. 4 paper; pH 3·6; 29 v/cm.; 2 hr. (see Table 2).

The four cystine peptides (α1, β, γ, and δ3) were oxidized and the resulting cysteic acid peptides fractionated by ionophoresis at pH 3·6 (Fig. 6). The results are summarized in Table 2. All four gave the same peptide (*b*, Fig. 6) which was neutral at pH 3·6 and was characterized by the presence of arginine and a large amount of glycine. Its structure can only be Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe.-Phe, identical with peptide *Bc*4.

The three acidic peptides α1a, βa and γa gave the same amino acids on hydrolysis but moved at different rates at pH 3·6. Band γa was by far the strongest and is probably $GluNH_2$.Leu.Glu.Asp-NH_2.Tyr.$CySO_3H$.$AspNH_2$ (*Ac*3) which would be expected to be a major component. Band α1a gave no ninhydrin reaction, suggesting that it was a pyrrolidonoyl derivative identical with γa except that the *N*-terminal glutamine residue had cyclized. This would account for α1 moving faster towards the anode than the other acidic cystine peptides as it has one less amino group. Band βa probably differs from γa only in having one amide group less and therefore being slightly more acidic.

On oxidation the only neutral cystine peptide (δ3) gave a neutral peptide (δ3b) identical with α1b, βb and γb, and an acidic peptide δ3a having the structure $CySO_3H$.$AspNH_2$ (*Ac*1).

Table 2. *Peptides from chymotryptic hydrolysate of insulin* (*Expt. Ic*)

Band (Figs. 4, 5, 6)	Amino acids of oxidized peptides	Probable structure
α1	a: $CySO_3H$, Asp, Glu, Tyr, Leu b: $CySO_3H$, Glu, Gly, Val, Leu, Phe, Arg	Pyr*.Leu.Glu.Asp(NH_2).Tyr.Cy.Asp(NH_2) S S Leu.Val.Cy.Gly.Glu.Arg.Gly.Phe.Phe
α2	Asp, Glu, Leu, Tyr (weak Val)	Glu(NH_2).Leu.Glu.Asp(NH_2).Tyr (*A*c5)
β	a: $CySO_3H$, Asp, Glu, Tyr, Leu b: $CySO_3H$, Glu, Gly, Val, Leu, Phe, Arg	See text
γ	a: $CySO_3H$, Asp, Glu, Tyr, Leu b: $CySO_3H$, Glu, Gly, Val, Leu, Phe, Arg	Glu(NH_2).Leu.Glu.Asp(NH_2).Tyr.Cy.Asp(NH_2) S S Leu.Val.Cy.Gly.Glu.Arg.Gly.Phe.Phe
δ1	Tyr, Phe	Phe.Tyr
δ2	Tyr, Phe	Phe + Tyr
δ3	a: $CySO_3H$, Asp b: $CySO_3H$, Glu, Gly, Val, Leu, Phe, Arg	Cy.Asp(NH_2) S S Leu.Val.Cy.Gly.Glu.Arg.Gly.Phe.Phe
ε	Thr, Ala, Tyr, Pro, Lys	Tyr.Thr.Pro.Lys.Ala (*B*c7)
ζ	Thr, Ala, Pro, Lys	Thr.Pro.Lys.Ala (*B*c5)
η	Ala, Pro, Lys	Pro.Lys.Ala

* Pyrrolidonoyl.

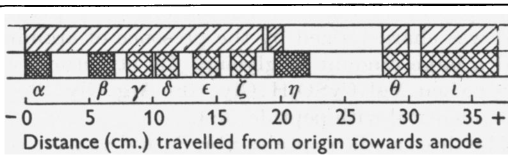


Fig. 7. Ionophoresis of crude 'tryptic' hydrolysate of insulin (Expt. *I*x). Soluble material from hydrolysate of 300 mg. insulin on 25 cm. wide no. 3 paper; pH 6·5; 33 v/cm.; 12 hr.

Hydrolysis with crude pancreatic extract (*Expt. I*x)

Fig. 7 shows the positions of the bands obtained by ionophoresis at pH 6·5 of the crude 'trypsin' hydrolysate. Band η, which had moved ahead of most of the ninhydrin-reactive material, was thought to be fairly pure and was oxidized directly. When part of the material from bands α–ζ and θ and ι was oxidized several cysteic acid peptides were obtained from most of the bands, indicating that they contained more than one cystine peptide.

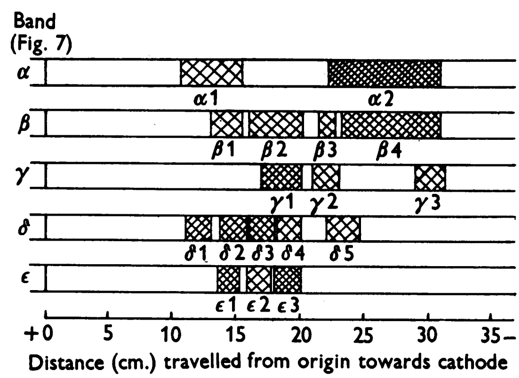


Fig. 8. Purification of bands α–ε from Expt. *I*x (Fig. 7) by ionophoresis: pH 3·6; 29 v/cm.; 6 hr.

The material from band ζ behaved in the same way as that from band η, and bands θ and ι were considered too weak to give any results after further manipulations, so these three bands were not further dealt with.

Table 3. *Cysteic acid peptides obtained from crude 'tryptic' hydrolysate of insulin* (*Expt. Ix*)

Band (Figs. 7–9)	Amino acids present in cysteic acid peptide	Assumed structure of cysteic acid peptide
β4a	$CySO_3H$, Glu, Gly, Val, Leu	Leu.Val.$CySO_3H$.Gly.Glu
β4b	$CySO_3H$, Asp	$CySO_3H$.$AspNH_2$
γ2a	$CySO_3H$, Gly, Val, Leu	Leu.Val.$CySO_3H$.Gly
γ2b	$CySO_3H$, Asp	$CySO_3H$.$AspNH_2$
δ1a	$CySO_3H$, Ser, Ala, Val	Same as ηb
δ1b	$CySO_3H$	Cysteic acid
δ2a	$CySO_3H$, Ser, Gly	?
δ2b	$CySO_3H$, Ser, Gly, Leu	Leu.$CySO_3H$.Gly.Ser
δ2c	$CySO_3H$, Ser, Ala, Val	Same as ηb
δ2d	$CySO_3H$, Ser, Gly	?
ηa	$CySO_3H$, Ser, Gly	$CySO_3H$.Gly.Ser
ηb	$CySO_3H$, Ser, Ala, Val	$CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$
ηc	$CySO_3H$, Ser, Gly, Ala	?

The remainder of the material from bands α–ε was purified by ionophoresis at pH 3·6. The separated cystine peptides, detected by the CN–nitroprusside reagent (Fig. 8) were eluted, oxidized and run again at pH 3·6 for the separation of the cysteic acid peptides whose positions are shown in Fig. 9.

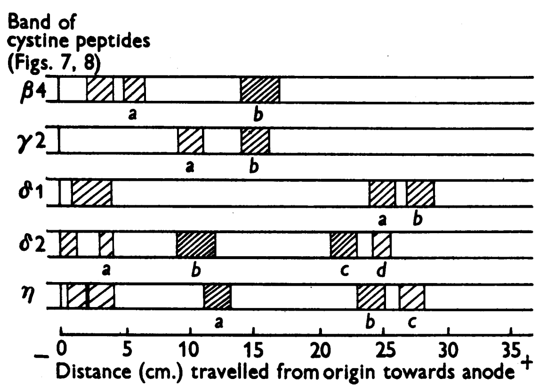


Fig. 9. Ionophoresis of cysteic acid peptides from Expt. *Ix* (Figs. 7, 8): pH 3·6; 29 v/cm., approx. 2 hr. Fractions β4, γ2 and δ1 were run for slightly longer than the other fractions (see Table 3).

The cysteic acid peptides strong enough to give results were eluted and hydrolysed, except in those cases where one of a pair of bands could be identified as cysteic acid by its speed on ionophoresis. The amino acids found in the hydrolysates and the probable identification of the cysteic acid peptides are shown in Table 3. Certain peptides (e.g. β2 and γ1) gave results indicating that they were probably complex mixtures and that no definite conclusions could be drawn from them. The results with these peptides are not recorded.

Peptides β4b and γ2b were identified as $CySO_3H$.$AspNH_2$ (identical with *A*c1). γ2a can only be Leu.Val.$CySO_3H$.Gly (positions *B* 17–20), since these four amino acids do not occur together elsewhere in the molecule, so that peptide γ2 must be

Leu.Val.Cy.Gly
S
S
Cy.$AspNH_2$.

By analogy it is concluded that β4 is

Leu.Val.Cy.Gly.Glu
S
S
Cy.$AspNH_2$,

though from its amino acid composition β4a could be Gly.Ileu.Val.Glu.Glu.$CySO_3H$ (positions *A* 1–6). Bands δ1a, δ2c and ηb all contain the same characteristic peptide which is present in large amounts in this hydrolysate. Judging from its ionophoretic mobility it is very acidic and has a high content of cysteic acid. In order to determine its structure it was subjected to partial hydrolysis.

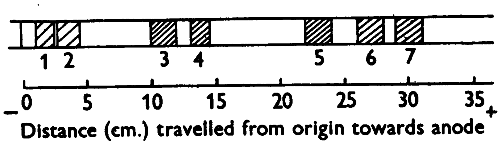


Fig. 10. Ionophoresis of partial hydrolysate of peptide *Ix*ηb: pH 3·6; 29 v/cm.; 2 hr.

Material from band ηb obtained from a digest of 135 mg. of insulin similar to that described above was incubated at 37° for 40 hr. in 11·7 N-HCl, and after removal of the HCl was subjected to ionophoresis at pH 3·6 for 2 hr. The positions of the bands detected by ninhydrin are shown in Fig. 10. A sample of cysteic acid moved at the same speed as band 6. The identities of the components of the various bands are given in Table 4. These show that ηb has the structure $CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$ (from positions *A* 6–11). It is probable that it does not contain the serine residue from position A 12, since such a peptide would be expected to give large amounts of free serine on acid hydrolysis, whereas only small amounts were obtained from peptide ηb.

Table 4. *Peptides obtained from partial hydrolysate of peptide Ixηb*

Band (Fig. 10)	Structure
1	Alanine
2	Serine
3	Ser.Val.$CySO_3H$ ($A\,1\zeta3$)
4	$CySO_3H$.Ala ($A\,1\gamma3$)
5	$CySO_3H$.$CySO_3H$.Ala ($A\,2\delta1$)
6	Cysteic acid
7	$CySO_3H$.$CySO_3H$

The other main cysteic acid peptide from the cystine peptide η is ηa([$CySO_3H$, Ser, Gly]). These three residues only occur together in positions B7–9 so that it must be $CySO_3H$.Gly.Ser. How ηb and ηa are linked together is not clear from these results since ηb contains three cysteic acid residues.

The two main oxidation products of peptide δ2 are $CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$ (δ2c) and δ2b which must be Leu.$CySO_3H$.Gly.Ser. Peptides δ2a, δ2d, and ηc were present in only small amounts and their composition is not certain. Their ionophoretic mobilities make it unlikely that δ2a and δ2b are $CySO_3H$.Gly.Ser (ηa).

Acid hydrolysis

In choosing conditions for acid hydrolysis a number of factors had to be considered. In Table 5 are listed the various peptides of cysteic acid encountered after oxidation of a partial hydrolysate

Table 5. *Cysteic acid peptides from oxidized partial hydrolysate of 'chymotryptic core' of insulin*

Peptide	Reference no. in previous papers	Distance moved towards anode on ionophoresis at pH 3·6 (Fig. 14) (cm.)
His.Leu.$CySO_3H$	$B\,1\gamma4$	−1·0
His.Leu.$CySO_3H$.Gly	$B\,4\beta1$	−1·0
Gly.Ileu.Val.Glu.Glu.$CySO_3H$	$A\,2\alpha7$	9·0
Leu.$CySO_3H$.Gly	$B\,1\alpha5$	10·0
Ser.Val.$CySO_3H$	$A\,1\zeta3$	10·5
$CySO_3H$.Ala	$A\,1\gamma3$	12·5
Leu.$CySO_3H$	$B\,1\alpha6$	13·5
Val.$CySO_3H$	$A\,1\zeta2$	13·5
Glu.Glu.$CySO_3H$	—	13·5
$CySO_3H$.Gly	$B\,1\alpha1$	14·5
Glu.$CySO_3H$	$A\,1\alpha1$	15·5
Glu.$CySO_3H$.$CySO_3H$.Ala	$A\,2\gamma1$	19·5
$CySO_3H$.$CySO_3H$.Ala	$A\,2\gamma1$	22·5
Glu.$CySO_3H$.$CySO_3H$	—	24·0
Cysteic acid	—	28·0
$CySO_3H$.$CySO_3H$	—	30·0

of the 'chymotryptic core'. From these it is possible to calculate that ninety-five different cystine peptides would be expected before oxidation. Of these, twelve contain a half-cystine residue (CyS) on one side of the —S.S— bond, and provide no information about the distribution of —S.S— bonds. Seventy-two contain the sequence Cy.Cy intact and are also of no use, whereas eleven contain a peptide sequence on both sides of the —S.S— bond and are of the type required in the present study. In order to avoid the very complicated mixture that would be produced if there was much of the Cy.Cy sequence left intact it was necessary to hydrolyse for as long as possible, but if the hydrolysis were carried out for too long the peptides containing a CyS residue would predominate and also there would be more danger of the interchange reaction occurring.

Using model systems it was shown that less interchange occurred in H_2SO_4 than in HCl of corresponding concentration (Ryle & Sanger, 1955). Although at high concentrations H_2SO_4 is less effective as a hydrolytic agent, it appeared that it would nevertheless be an advantage to use it. Insulin is insoluble in aqueous H_2SO_4 so that it was necessary to add acetic acid to dissolve it.

In an initial experiment 3·7 N-H_2SO_4 in 25 % (v/v) acetic acid was used. After 2 days at 37° a precipitate started to form and was separated after 5 days. This 'acid core' represented about 30–40 % of the insulin and on oxidation gave the peptides Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala and Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly, besides larger peptides which were not identified. It seems to be a mixture of large peptides which are less soluble than insulin. It could also be precipitated with water from a hydrolysate that had been obtained by the action of 5 N-H_2SO_4 in 50 % (v/v) acetic acid for 1 day at 37°, so its formation was not caused by a mass action effect due to its insolubility.

In later experiments 10 N-H_2SO_4 in 50 % (v/v) acetic acid at 37° was used for hydrolysis. The rate of hydrolysis of insulin in this medium is given in Table 6.

Table 6. *Rate of hydrolysis of insulin in 10 N sulphuric acid*

Amino N (Van Slyke nitrous acid method, 11 min. reaction) as % of value after hydrolysis for 48 hr. at 105° (see Peters & Van Slyke, 1932).

Reagent	Temp. (°)	Time	% hydrolysis
10 N-H_2SO_4 in 50 % (v/v) acetic acid	37	4 days	26
		10 days	37
		14 days	49
10 N-H_2SO_4 in 30 % (v/v) acetic acid	100	23 min.	40
		35 min.	66
		60 min.	80
		11 hr.	105

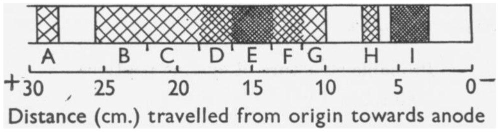


Fig. 11. Ionophoresis of fraction $I1\alpha$: 90 mg. on 20 cm. wide no. 3 paper; pH 6·5; 33 v/cm.; 1·75 hr.

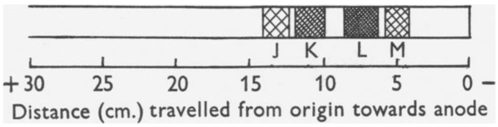


Fig. 12. Fractionation of band $I1\alpha$I (Fig. 11) by ionophoresis: no. 3 paper; pH 6·5; 33 v/cm.; 12 hr.

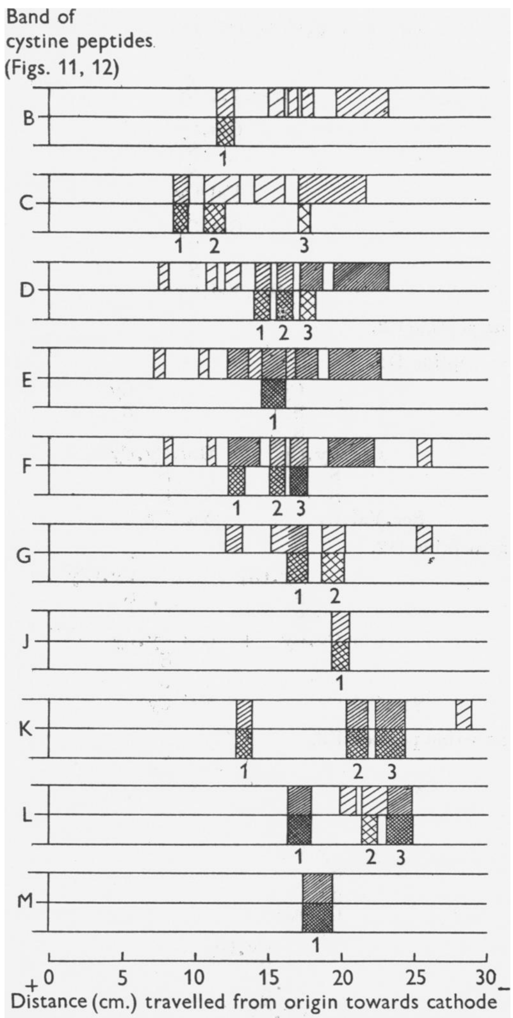


Fig. 13. Further ionophoretic fractionation of peptides from $I1\alpha$ (Figs. 11, 12): pH 3·6; 29 v/cm.; 6 hr.

To obtain sufficiently simple peptides at 37° it was necessary to continue the hydrolysis for at least 14 days, so in some experiments to save time a temperature of 100° was used. The corresponding rates of breakdown are also shown in Table 6. There was slightly more disulphide interchange at this higher temperature, but it did not significantly affect the results (see Ryle & Sanger, 1955).

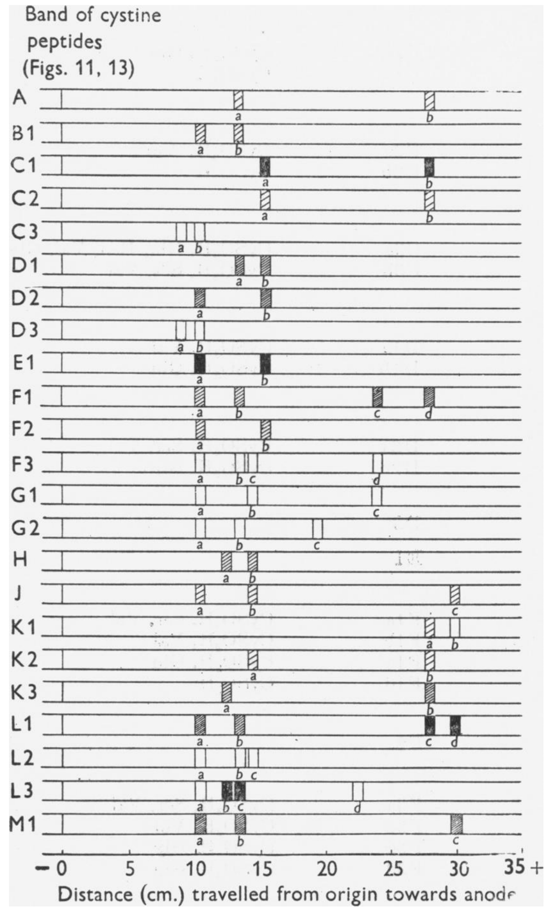


Fig. 14. Ionophoresis of cysteic acid peptides from $I1\alpha$ (Figs. 11–13): no. 4 paper; pH 3·6; 29 v/cm.; 2 hr. (see Table 7). All bands coloured with ninhydrin.

Experiment I1. Using Amberlite IR-4B at pH 3–4 the hydrolysate was separated into two fractions $I1\alpha$ and $I1\beta$. The former, which was adsorbed on the column, contained acidic and some neutral cystine peptides, whereas fraction $I1\beta$ contained the remaining neutral and basic peptides. Most of the neutral non-cystine peptides were in fraction $I1\beta$ as would be expected (Consden, Gordon & Martin, 1948).

Table 7. *Peptides from fraction I1α*

Band (Fig. 14)	Amino acids of oxidized peptides	Probable structure of cystine peptides
Aa Ab	CySO$_3$H, Glu CySO$_3$H	Glu.Glu.Cy S S Cy
B1a B1b	CySO$_3$H, Ser, Val CySO$_3$H, Glu	Glu.Glu.Cy S S Ser.Val.Cy
C1a C1b	CySO$_3$H, Glu CySO$_3$H	Glu.Cy S S Cy
C2a C2b	CySO$_3$H, Asp CySO$_3$H	Cy S S Cy.Asp
C3a C3b	CySO$_3$H, Glu, Ileu, Val, Gly CySO$_3$H, Ser, Val	Gly.Ileu.Val.Glu.Glu.Cy S S Ser.Val.Cy
D1a D1b	CySO$_3$H, Val CySO$_3$H, Glu	Glu.Cy S S Val.Cy
D2a D2b	CySO$_3$H, Ser, Val CySO$_3$H, Glu	Glu.Cy S S Ser.Val.Cy
D3		Same as peptide C3.
E1		Same as peptide D2.
F1a F1b F1c F1d	CySO$_3$H, Ser, Val CySO$_3$H, Val CySO$_3$H, Glu CySO$_3$H	Cy Cy S S S S Glu.Cy.Cy + Glu.Cy.Cy S S S S Ser.Val.Cy Val.Cy
F2		Same as peptide D2.
F3a F3b F3c F3d	CySO$_3$H, Ser, Val CySO$_3$H, Val CySO$_3$H, Gly CySO$_3$H, Glu	Cy.Gly Cy.Gly S S S S Glu.Cy.Cy + Glu.Cy.Cy S S S S Ser.Val.Cy Val.Cy
G1		Same as serine peptide F3.
G2a G2b G2c	CySO$_3$H, Ser, Val CySO$_3$H, Glu, Val CySO$_3$H, Glu, Ala	?
Ha Hb	CySO$_3$H, Ala CySO$_3$H, Gly	Cy.Gly S S Cy.Ala
Ja Jb Jc	CySO$_3$H, Ser, Val CySO$_3$H, Gly CySO$_3$H	Cy.Gly S S Cy.Cy S S Ser.Val.Cy

Table 7 (*cont.*)

Band (Fig. 14)	Amino acids of oxidized peptides	Probable structure of cystine peptides
K1a K1b	$CySO_3H$ $CySO_3H$	?
K2a K2b	$CySO_3H$, Gly $CySO_3H$	Cy.Gly S S Cy
K3a K3b	$CySO_3H$, Ala $CySO_3H$	Cy S S Cy.Ala
L1a L1b L1c L1d	$CySO_3H$, Ser, Val $CySO_3H$, Val $CySO_3H$ $CySO_3H$	Cy S S Cy.Cy S S Ser.Val.Cy + Cy S S Cy.Cy S S Val.Cy
L2a L2b L2c	$CySO_3H$, Ser, Val $CySO_3H$, Val, Leu $CySO_3H$, Gly	?
L3a L3b L3c L3d	$CySO_3H$, Ser, Val $CySO_3H$, Ala $CySO_3H$, Leu $CySO_3H$, Ala	Mainly Leu.Cy S S Cy.Ala (*I*2H)
M1a M1b M1c	$CySO_3H$, Ser, Val $CySO_3H$, Leu (weak Val) $CySO_3H$	Leu.Cy S S Cy.Cy S S Ser.Val.Cy + Leu.Cy S S Cy.Cy S S Val.Cy

Fig. 11 shows the ionophoretic separation of fraction *I*1α at pH 6·5. The main neutral band *I*1αI was eluted and refractionated at the same pH for a longer time (12 hr.) when it separated into a number of different fractions (Fig. 12). The acidic cystine peptides (*B–G*) were not adequately separated and were refractionated by ionophoresis at pH 3·6, as also were most of the neutral peptides (Fig. 13). Fig. 14 shows the ionophoretic separations at pH 3·6 of the oxidation products of the various cystine peptides. Not all the ionophoreses were in fact run for exactly 2 hr. but the diagram has been drawn as if they were, so that all samples of a particular peptide are found on the same vertical line. After ionophoresis the various peptides were identified by their amino acid composition and the results are given in Table 7.

The various peptides encountered in this work are listed in Table 5 together with the distance they move on ionophoresis at pH 3·6 under the conditions of Fig. 14. A number of these peptides had not been encountered in previous work with the oxidized chains, and were only revealed by the more efficient ionophoretic method used here. Thus Glu.Glu.$CySO_3H$ (*I*1αAa) was identified as a peptide containing a high proportion of glutamic acid with cysteic acid and moving considerably slower on ionophoresis than Glu.$CySO_3H$. Glu.$CySO_3H$.$CySO_3H$ (*I*1αF1c, *I*1αF3d) was a peptide moving at the characteristic rate of peptides having two residues of cysteic acid, and $CySO_3H$.$CySO_3H$ (*I*1αL1d, *I*1αM1c, etc.) was found as a band moving faster than free cysteic acid but giving only cysteic acid on hydrolysis.

From their ionophoretic rates and composition it was possible to identify the cysteic acid peptides, and the structures of the cystine peptides given in Table 7 were deduced from them. Where a peptide with two cysteic acid residues was produced on oxidation it was not possible to deduce the structure of the original cystine peptide from the experimental results and the formulae given were derived when the structure of insulin was known.

During the purification of the cystine peptides at pH 3·6 (Fig. 13), weak CN–nitroprusside-reacting bands were frequently observed, moving towards the anode or slowly towards the cathode. Their acidity suggested that they were cysteic acid peptides. As they also contained an intact —S.S— bond, they were probably derived from the peptides containing the Cy.Cy sequence by partial oxidation during the purification procedure. $I1\alpha$K1 is probably such a peptide. The others were too faint to investigate.

Fig. 15 shows the ionophoresis of fraction $I1\beta$.

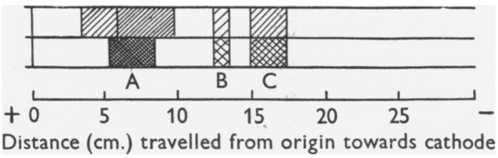


Fig. 15. Ionophoresis of fraction $I1\beta$: no. 3 paper; pH 6·5; 33 v/cm.; 6 hr.

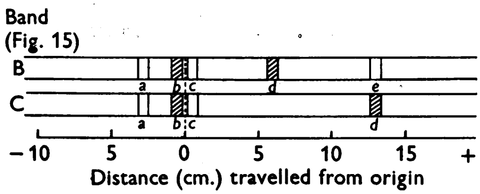


Fig. 16. Ionophoresis of oxidation products of fractions $I1\beta$B and $I1\beta$C (Fig. 15): no. 4 paper; pH 3·6; 29 v/cm.; 1 hr. (see Table 8).

The two basic peptides B and C were not present in sufficient concentration to refractionate, so were oxidized directly and subjected to ionophoresis at pH 3·6 (Fig. 16). The results are given in Table 8. It is probable that neither of these were pure but consisted of the peptides shown in Table 8.

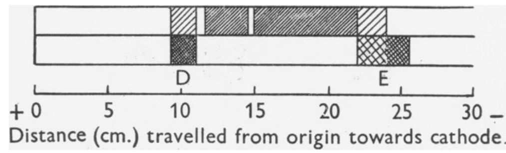


Fig. 17. Ionophoresis of fraction $I1\beta$A (Fig. 15): no. 3 paper; pH 3·6; 29 v/cm.; 7 hr.

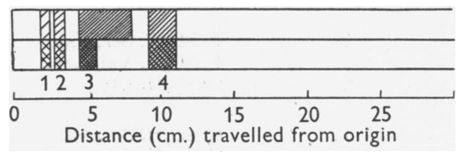


Fig. 18. One-dimensional chromatogram of fraction $I1\beta$E (Fig. 17): no. 3 paper; *n*-butanol–acetic acid–water (4:1:5, by vol.). Run for 36 hr. at 25° and solvent allowed to drip off the bottom of the paper.

Table 8. *Peptides from fraction* $I1\beta$

Band (Figs. 16, 19)	Amino acids of oxidized peptides	Structure of cystine peptide
Ba	No $CySO_3H$	His.Leu.Cy His.Leu.Cy.Gly S + S S S Cy.Ala Cy.Ala
Bb	$CySO_3H$, Gly, His, Leu	
Bc	No $CySO_3H$	
Bd	$CySO_3H$, Ala	
Be	$CySO_3H$	
Ca	No $CySO_3H$	His.Leu.Cy His.Leu.Cy.Gly S + S S S Cy Cy
Cb	$CySO_3H$, His, Leu, (weak Gly)	
Cc	No $CySO_3H$	
Cd	$CySO_3H$	
D	$CySO_3H$	Cy S S Cy
E1a	$CySO_3H$, Glu, Ser, Gly (all very weak)	?
E1b	$CySO_3H$	
E2a*	$CySO_3H$, Ala	Cy S S Ala.Cy
E2b	$CySO_3H$	
E3a	$CySO_3H$, Ser, Val	Cy S S Ser.Val.Cy
E3b	$CySO_3H$	
E4a	$CySO_3H$, Leu	Leu.Cy S S Cy
E4b	$CySO_3H$	

* Peptide E2a moved considerably faster on ionophoresis at pH 3·7 (Fig. 19) than $CySO_3H$.Ala (e.g. in peptide $I1\alpha$K3a). It seems probable that it is Ala.$CySO_3H$ formed by inversion during hydrolysis. This reaction has in fact been shown to occur under similar conditions (Tuppy & Bodo, 1954).

The main neutral band $I1\beta$A was refractionated by ionophoresis at pH 3·6 (Fig. 17) to give two bands D and E. D was free cystine, whereas E was further fractionated by one-dimensional paper chromatography in butanol–acetic acid (Fig. 18). Fig. 19 shows the ionophoresis of the oxidized peptides and the results are listed in Table 8.

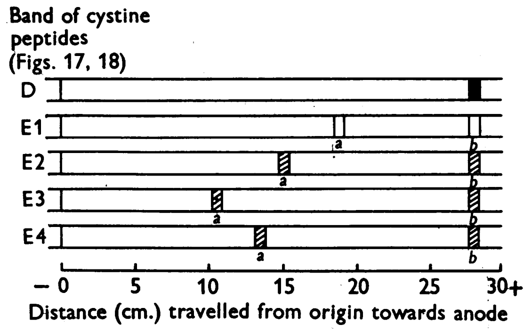


Fig. 19. Ionophoresis of oxidized peptides from $I1\beta$A (Figs. 15, 17, 18): no. 4 paper; pH 3·6; 29 v/cm.; 2 hr. (see Table 8). All bands coloured with ninhydrin.

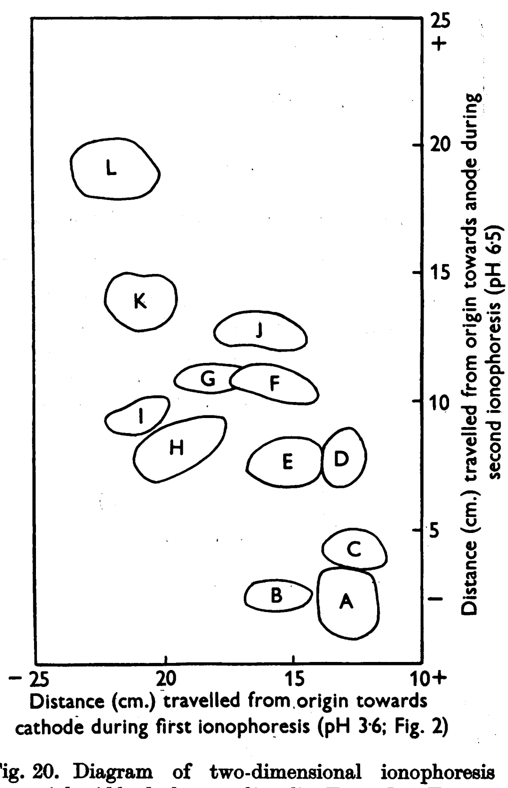


Fig. 20. Diagram of two-dimensional ionophoresis of partial acid hydrolysate of insulin (Expt. $I2$). First run as shown in Fig. 2. Second run: pH 6·5; 33 v/cm.; 10 hr.; spots located by CN–nitroprusside test.

Experiment I2. Fig. 20 shows the distribution of the cystine peptides in the two-dimensional ionophoresis experiment, and Fig. 21 is a diagram of the ionophoreses of their oxidation products at pH 3·6 showing the distribution of the cysteic acid peptides produced. Some of these were eluted, hydrolysed and their amino acid composition determined. The results and the deductions made are summarized in Table 9. The probable structure of most of the other peptides could be deduced from the position of the cysteic acid peptides on the pH 3·6 ionophoresis.

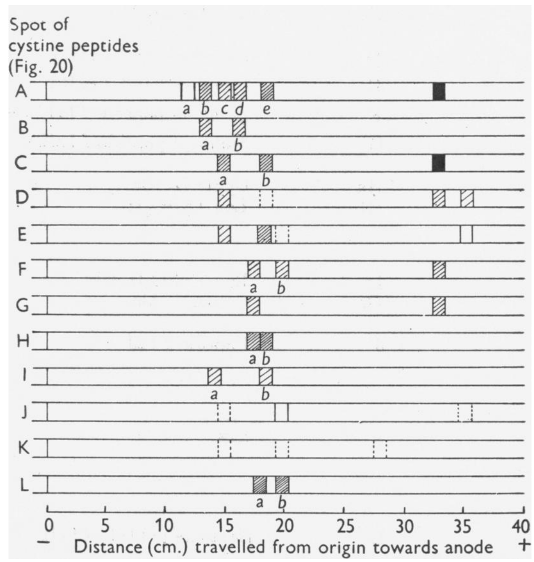


Fig. 21. Ionophoresis of cysteic acid peptides from Expt. $I2$: no. 4 paper; pH 3·6; 29 v/cm.; 2·3 hr. (see Table 9).

DISCUSSION

A good fractionation of neutral cystine peptides was obtained by ionophoresis at pH 6·5 for a relatively long time (Figs. 12, 20). This would appear to be due to the unusually low pK of the amino groups of cystine residues in peptides. Greenstein, Klemperer & Wyman (1939) found values of 6·36 and 6·95 for the pK' values of the amino groups of cystylbisglycine, whereas the corresponding values for bisglycylcystine were more normal (7·94 and 7·94). Thus peptides such as

Cy.Ala
S
S (Fig. 20, spot L)
Cy.Gly

are somewhat acidic at pH 6·5 owing to the amino groups being partially discharged and

Table 9. *Peptides from Expt. I2*

Band (Figs. 20, 21)	Structure of cysteic acid peptide	Structure of cystine peptide
Aa Ab Ac Ad Ae	Tyr.Leu.Val.$CySO_3H$ (*B*2α21) Leu.Val.$CySO_3H$ (*B*1α8) Ser.Val.$CySO_3H$ Tyr.$CySO_3H$ (*A*1a7) Leu.$CySO_3H$ + Val.$CySO_3H$	Mixture of peptides of type Cy S S X.Cy
Ba Bb	Leu.Val.$CySO_3H$ (*B*1α8) Tyr.$CySO_3H$ (*A*1a7)	Leu.Val.Cy S S Tyr.Cy
Ca Cb	Ser.Val.$CySO_3H$ Val.$CySO_3H$	Peptide *I*1βE3 + Val.Cy S S Cy
D	Same as band *I*1αL1	
E	Same as band *I*1αM1	
F	Same as peptide *I*1αK2 + *I*1αK3	
G	Same as peptide *I*1αK3	
Ha Hb	$CySO_3H$.Ala Leu.$CySO_3H$	Cy.Ala S S Leu.Cy
Ia Ib	Leu.$CySO_3H$.Gly $CySO_3H$.Ala	Cy.Ala S S Leu.Cy.Gly
J K L	Peptide *I*1αJ —* Same as peptide *I*1αH	Ser.Val.Cy S S Probably Cy.Cy S S Cy.Gly

* The cysteic acid peptides were not hydrolysed, but the structure was deduced from their ionophoretic rates.

move relatively rapidly on ionophoresis, peptides such as

Cy.Ala
S
S (Fig. 20, spot H)
Leu.Cy

containing only one free amino group of cystine are less acidic, whereas those like

Leu.Val.Cy
S
S (Fig. 20, spot B)
Tyr.Cy

are almost neutral. In this way the neutral cystine peptides could largely be separated from each other and also from other neutral peptides not containing cystine.

Two main cystine-containing peptides (*I*cδ3 and *I*cγ, Table 2) were present in the water-soluble fraction of the chymotryptic hydrolysate. Their oxidation products had been encountered in studies on the fractions *A* and *B*, and their structures, which are shown in Table 2, establish the presence of a disulphide bridge joining the half-cystine residues in positions *A* 20 and *B* 19. This is confirmed by the results with the crude 'tryptic' hydrolysate, in which

Leu.Val.Cy.Gly
S
S (*I*xγ2)
Cy.$AspNH_2$

and

Leu.Val.Cy.Gly.Glu
S
S (*I*xβ4)
Cy.$AspNH_2$

were detected and with the acid hydrolysate in which

Leu.Val.Cy
S
S (*I*2B)
Tyr.Cy

was found. In the chymotryptic hydrolysate, the remaining cystine residues are present in the insoluble 'core' and in the crude 'tryptic' hydrolysate they are present in peptides $Ix\eta$ and $Ix\delta2$ in which CyS.Gly.Ser and Leu.CyS.Gly.Ser respectively are coupled with CyS.CyS.Ala.Ser.Val.-CyS. Neither enzyme preparation split between the three CyS residues of the glycyl chain.

A number of neutral peptides from the acid hydrolysates gave $CySO_3H$.Ala on oxidation. These were:

Cy.Gly
S
S (*I*1αH)
Cy.Ala

Leu.Cy
S
S (*I*2H)
Cy.Ala

and

Leu.Cy.Gly
S
S (*I*2I).
Cy.Ala

$CySO_3H$.Ala was found in the basic fraction *I*1βB linked with His.Leu.$CySO_3H$ and His.Leu.-$CySO_3H$.Gly. All these peptides establish that there is a disulphide bond between positions *A* 7 and *B* 7.

This only leaves the half-cystine residues in positions *A* 6 and *A* 11 to be considered and they must therefore be joined together. This is proved by the presence of relatively high concentrations of the acidic peptides

Ser.Val.Cy
S
S (*I*1αD2)
Glu.Cy

and

Val.Cy
S
S (*I*1αD1)
Glu.Cy

and confirmed by the peptides

Glu.Glu.Cy
S
S (*I*1αB1)
Ser.Val.Cy

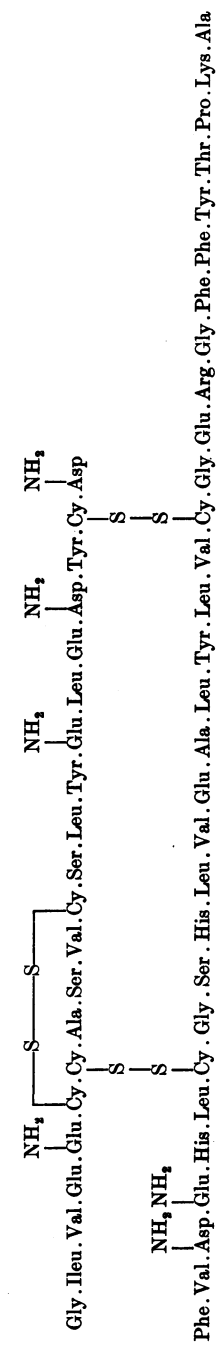
and

Gly.Ileu.Val.Glu.Glu.Cy
S
S (*I*1αC3)
Ser.Val.Cy

A considerable number of peptides containing the Cy.Cy sequence were detected. Since their structures could all be explained on the basis of the above results, they added confirmation and the fact that no peptides were detected which could not be explained showed that no appreciable disulphide interchange had occurred during the experiments.

The results are summarized in Table 10, which gives the complete structure of insulin. This structure contains a ring, in which the disulphide bond in positions *A* 6–*A* 11 is included. Clearly to form such a bond the polypeptide chain must be folded in such a way that the two half-cystine residues are close together and this fact must be

Table 10. *The structure of insulin*



taken into account when considering possible configurations for the polypeptide chain in this area. It is interesting that the ring is the same size as the similar disulphide ring found in oxytocin (Tuppy & Michl, 1953; du Vigneaud, Ressler & Trippett, 1953) and vasopressin (Acher & Chauvet, 1953; du Vigneaud, Lawler & Popenoe, 1953), which suggests that it may have a possible structural or biological significance.

SUMMARY

1. Insulin was subjected to partial hydrolysis with chymotrypsin, with a crude pancreatic extract and with acid under conditions in which the disulphide bonds were stable.

2. Cystine-containing peptides in the hydrolysates were separated and their structure determined after oxidation to cysteic acid peptides. Paper ionophoresis at high potential gradients in pyridine-acetic acid buffers was found useful for the separations.

3. From the structure of the cystine peptides the distribution of the disulphide bonds of insulin was deduced and is shown in Table 10.

A.P.R. is indebted to the Medical Research Council for a Scholarship for training in research methods.

REFERENCES

Acher, R. & Chauvet, J. (1953). *Biochim. biophys. Acta*, **12**, 487.
Acher, R. & Crocker, C. (1952). *Biochim. biophys. Acta*, **9**, 704.
Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. L. (1948). *Biochem. J.* **42**, 116.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1948). *Biochem. J.* **42**, 443.
Craig, L. C., Gregory, J. D. & Hausmann, W. (1950). *Analyt. Chem.* **22**, 1462.
Du Vigneaud, V., Lawler, H. C. & Popenoe, E. A. (1953). *J. Amer. chem. Soc.* **75**, 4880.
Du Vigneaud, V., Ressler, C. & Trippett, S. (1953). *J. biol. Chem.* **205**, 949.
Fredericq, E. (1953). *Nature, Lond.*, **171**, 570.
Greenstein, J. P., Klemperer, F. W. & Wyman, J. (1939). *J. biol. Chem.* **129**, 681.
Harfenist, E. J. & Craig, L. C. (1952). *J. Amer. chem. Soc.* **74**, 3087.
Kupke, D. W. & Linderstrøm-Lang, K. (1954). *Biochim. biophys. Acta*, **13**, 153.
Michl, H. (1951). *Mh. Chem.* **82**, 489.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*. Vol. 2, p. 385. London: Baillière, Tindall and Cox.
Ryle, A. P. & Sanger, F. (1955). *Biochem. J.* **60**, 535.
Sanger, F. (1949*a*). *Biochem. J.* **44**, 126.
Sanger, F. (1949*b*). *Biochem. J.* **45**, 563.
Sanger, F., Smith, L. F. & Kitai, R. (1954). *Biochem. J.* **58**, vi.
Sanger, F. & Thompson, E. O. P. (1953*a*). *Biochem. J.* **53**, 353.
Sanger, F. & Thompson, E. O. P. (1953*b*). *Biochem. J.* **53**, 366.
Sanger, F., Thompson, E. O. P. & Kitai, R. (1955). *Biochem. J.* **59**, 509.
Sanger, F. & Tuppy, H. (1951*a*). *Biochem. J.* **49**, 463.
Sanger, F. & Tuppy, H. (1951*b*). *Biochem. J.* **49**, 481.
Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
Tuppy, H. & Bodo, G. (1954). *Mh. Chem.* **85**, 807.
Tuppy, H. & Michl, H. (1953). *Mh. Chem.* **84**, 1011.

The Structure of Pig and Sheep Insulins

BY H. BROWN,* F. SANGER† AND RUTH KITAI
Biochemical Laboratory, University of Cambridge

(*Received* 26 *November* 1954)

Insulins from a variety of different animal species show the same biological activity (Scott & Fisher, 1940) and immunological behaviour (Wasserman & Mirsky, 1942). They have the same crystalline form and mixtures of different insulins behave as a single substance in the phase-rule solubility test (Lens & Evertzen, 1952). However, in a preliminary chemical study of pig and sheep insulin it was shown that, whereas the general structure was similar, there were certain differences in individual amino acid residues (Sanger, 1949*b*). Harfenist & Craig (1952) have recently analysed the different insulins, and found differences in the contents of the amino acid residues given in Table 1. No differences were found for the other amino acids.

The complete sequence of amino acids in cattle insulin has recently been determined in this laboratory (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*), and the present paper describes similar studies on pig and sheep insulins. Since the larger part of the molecule was the same for all three species it was justifiable not to determine the amino acid sequence unequivocally in each case, but only to identify lower peptides embodying each residue in the molecule. Where

* Damon Runyon Cancer Research Fellow. Present address: Department of Surgery, University of Wisconsin, U.S.A.

† Member of the Scientific Staff of the Medical Research Council.